Methylation of the *PTEN* promoter defines low-grade gliomas and secondary glioblastoma

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Glioblastoma multiforme (GBM) can present as either de novo or secondary tumors arising from previously diagnosed low-grade gliomas. Although these tumor types are phenotypically indistinguishable, de novo and secondary GBMs are associated with distinct genetic characteristics. *PTEN* mutations, which result in activation of the phosphoinositide 3-kinase (PI3K) signal transduction pathway, are frequent in de novo but not in secondary GBMs or their antecedent low-grade tumors. Results we present here show that grade II astrocytomas, oligodendrogliomas, and oligoastrocytomas commonly display methylation of the *PTEN* promoter, a finding that is absent in nontumor brain specimens and rare in de novo GBMs. Methylation of the *PTEN* promoter correlates with protein kinase B (PKB/Akt) phosphorylation, reflecting functional activation of the PI3K pathway. Our results also demonstrate frequent methylation of the *PTEN* promoter in grade III astrocytomas and secondary GBMs, consistent with the hypothesis that these tumors arise from lower grade precursors. *PTEN* methylation is rare in de novo GBMs and is mutually exclusive with *PTEN* mutations. We conclude that methylation of the *PTEN* promoter may represent an alternate mechanism by which PI3K signaling is increased in grade II and III gliomas as well as secondary GBMs, a finding that offers new therapeutic approaches in these patients. *Neuro-Oncology 9, 271–279, 2007 (Posted to Neuro-Oncology [serial online], Doc. D06-00100, May 15, 2007. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2007-003)*

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Elucidation of genetic alterations present in human cancers has the potential to direct the choice of therapeutic modalities. A growing number of molecularly targeted agents in clinical practice target pathways that underlie tumor genesis and progression. By example, the ABL/PDGFR/KIT inhibitor imatinib induces potent responses of tumors that express activated forms of ABL,[1] platelet-derived growth factor receptor (PDGFR),[2] or KIT.[3] Similarly, epidermal growth factor receptor (EGFR) inhibitors, including gefitinib and erlotinib, have dramatic antineoplastic effects in lung tumors that express mutated forms of the EGFR[4,5] or lung cancers and GBMs that display *EGFR* gene amplification.[6–8] Current clinical trials are testing a rapidly expanding number of kinase inhibitors. Clinical efficacy of these drugs will likely prove greatest in early-stage tumors that

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may harbor fewer genetic aberrations. Thus, although kinase aberrations appear more common in advanced tumors, interrogation of genetic alterations that incur increased kinase activity is critical in early-stage, low-grade malignancies, as well. Such exploration will likely increase the as-of-yet limited therapeutic options available for patients with low-grade gliomas.

Glioblastoma (GBM) is a highly malignant brain tumor that is uniformly fatal. Efforts to improve the surgical, radiotherapeutic, and chemotherapeutic approaches to glioma treatment have failed to substantially increase long-term disease control.[9] Although standard antineoplastic therapies have produced little clinical progress, genetic analyses of gliomas have increased our understanding of the molecular pathogenesis of these tumors.[10] GBMs can either arise from low-grade gliomas or present as de novo tumors. Low-grade tumors can arise as astrocytomas or oligodendrogliomas, which may depend on the cell type that sustained the initiating oncogenic alterations, or a mixed population termed oligoastrocytoma (OA). These two presentations of GBM have distinct, albeit overlapping, genetic alterations. While *EGFR* gene amplification and *PTEN* mutations are common in de novo GBMs, both of these alterations are infrequent in secondary GBMs. Conversely, secondary GBMs commonly contain *TP53* mutations and *PDGFR* gene amplifications, aberrations that are less frequent in de novo GBMs.[11]

The significant proportion of de novo GBMs harboring *PTEN* mutations, 14%–47%,[12,13] attests to the importance of this mutation in glioma pathogenesis. Moreover, low PTEN RNA levels,[14,15] low protein levels,[16] and loss of heterozygosity (LOH)[17] each portends decreased survival in GBM patients. PTEN exerts its effects by dephosphorylating the phospholipid second messenger phosphatidylinositol (3,4,5)trisphosphate (PtdIns[3,4,5]P3) that is generated by phosphoinositide 3-kinase (PI3K).[18] PtdIns(3,4,5)P3 recruits proteins that contain pleckstrin homology domains, such as protein kinase B (PKB/Akt), to the plasma membrane, allowing its phosphorylation and activation by another protein kinase, 3'-phosphoinositide-dependent kinase-1 (PDK1).[19] Activation of PKB/Akt and other targets of PtdIns(3,4,5)P3 promotes a number of biological events important in tumorigenesis, including proliferation, survival, migration, and angiogenesis.[20]

Although *PTEN* mutations are exceedingly rare in low-grade gliomas, these tumors exhibit lower PTEN protein levels than does normal brain. Consequently, low-grade gliomas also display elevated levels of PKB/Akt phosphorylation and activity.[16] We therefore hypothesized that methylation of the *PTEN* promoter may underlie decreased PTEN expression in tumors without *PTEN* mutations. Results we present here show that low-grade gliomas exhibit frequent *PTEN* promoter methylation, with a significantly lower frequency observed in de novo GBM specimens. In contrast, the *PTEN* promoter is frequently methylated in secondary GBMs. No *PTEN* promoter methylation was seen in 13 samples of normal brain, indicating that *PTEN* promoter methylation may represent an epigenetic mechanism to reduce PTEN expression during tumorigenesis. *PTEN* methylation in low-grade gliomas was associated with increased PKB/Akt phosphorylation, consistent with the known role of PTEN in regulating the PI3K/PKB/Akt pathway. We discuss the implications of these findings for our understanding of the genetics of de novo and secondary GBMs, as well as for therapeutic alternatives for low-grade gliomas.

Materials and Methods

Patients and Tissue Samples

Frozen tissue and formalin-fixed, paraffin-embedded sections of 23 de novo GBMs, 11 secondary GBMs, 14 grade II astrocytomas, 15 grade II OAs, eight oligodendrogliomas, 19 grade III astrocytomas, and 13 samples of nontumor brain were obtained from the University of California, San Francisco Brain Tumor Research Center tissue bank, under appropriate institutional review board approval. De novo GBM patients were 35–76 years old (mean = 61 years, n = 23), secondary GBM patients were 23–50 years old (mean = 37 years, n = 11), OA patients were 24–48 years old (mean = 33 years, n = 15), grade II astrocytoma patients were 21–50 years old (mean = 36 years, n = 14), grade II oligodendroglioma patients were 27–51 years old (mean = 41 years, n = 8) grade III OA patients were 26–51 years old (mean = 39 years, n = 10), and grade III astrocytoma patients were 23–61 years old (mean = 43 years, n = 10). Tumor samples were defined as secondary GBM if the patients had prior histological diagnosis of a low-grade glioma. All but two patients were documented as having radiation and/or chemotherapy prior to progression to GBM, and the remaining two patients had their initial diagnosis 14 and 45 months prior to the GBM surgery that provided tissue analyzed in this study. All ages are given at the time of surgery, which occurred at the University of California, San Francisco between 1990 and 2003.

DNA Preparation and Bisulfite Treatment

Genomic DNA was isolated from approximately 25 mg wet weight of each frozen tissue sample using QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions and eluted twice in a total of 100 μl of elution buffer. This procedure yielded 5–40 μg of DNA. Bisulfite modification of genomic DNA was performed as described previously.[21] Briefly, 1 μg purified DNA was diluted in 36 μl H_2O, 4 μl 3.0 M NaOH was added, and DNA was denatured at 37°C for 15 min. The samples were then treated with 416 μl 3.6 M sodium bisulfite solution (pH 5.0) and 24 μl of 10 mM hydroquinone. All solutions were prepared fresh for each analysis. Samples were incubated at 55°C for 16 h. Two drops of mineral oil were layered on top of the solution to prevent evaporation. Bisulfite-modified DNA was purified with the Wizard DNA Clean-up System and vacuum manifold (Promega, Madison, WI, USA) according to the manufacturer's manual. Freshly

prepared NaOH solution was added to a final concentration of 0.3 M, and samples were incubated at 37°C for 15 min, followed by neutralization with ammonium acetate (pH 7.0; final concentration, 3.0 M) and ethanol precipitation. Normal human peripheral blood lymphocyte DNA samples, treated and untreated with DNA methylase (M. Sss I; New England BioLabs, Beverly, MA, USA), were also modified as positive and negative controls, respectively.

Methylation-Specific PCR

To examine whether the *PTEN* promoter is methylated in glioma specimens, we used methylation-specific primers that had previously been used to demonstrate methylation of the *PTEN* promoter in a subset of non–small-cell lung cancer samples.[22] These primers amplify a 181–base pair region of the *PTEN* promoter that starts 2,477 nucleotides upstream from the translation start site—methylated primers: forward, 5'-GTTTGGGGATTTTTTTTTCGC-3'; reverse, 5'-AACCCTTCCTACGCCGCG-3'; unmethylated primers: forward, 5'-TATTAGTTTGGGGATTTTTTTTTTGT-3'; reverse, 5'-CCCAACCCTTCCTACACCACA-3'. It should be noted that these primers do not amplify the highly homologous *PTEN* pseudogene located on chromosome 9p21, as these sequences lie outside the region of similarity.

The 25 μl PCR reaction contained 25 ng bisulfite DNA, 2% dimethylsulfoxide, 1.5 mM $MgCl_2$, and 1 U Ampli Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). PCR reactions were cycled in a Gene Amp 2700 thermocycler (Applied Biosystems) under the following conditions: preheat at 95°C for 10 min, 95°C for 30 s, 62°C for 30 s, 72°C for 30 s, 38 cycles, and a final extension at 72°C for 7 min. Aliquots (12 μl) of methylation-specific PCR (MSP) products were analyzed on 3% agarose gels, stained with ethidium bromide, and visualized under UV illumination. Results were recorded with a digital imaging system. For each PCR experiment, DNA from peripheral blood of normal blood donors treated with and without CpG methylase and bisulfite was included as positive and negative controls, respectively. We repeated MSP assays on all samples and found no discordant results among replicates. The MSP assay is sensitive to approximately 5% methylated product. To confirm the efficiency of the bisulfite modification and the specificity of MSP, bisulfite sequencing of the PCR products was carried out using the procedure reported previously.[23]

PTEN *Sequencing*

Fifty nanograms of genomic DNA was used for 40 rounds of PCR using the *PTEN* exon primers listed in Table 1. Primers that span the exon/intron boundaries were designed using the algorithm Primer3 (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

PCR products were then sequenced in both directions on an ABI3700 DNA analyzer (Applied Biosystems), using the same primers used for amplification.

Western Blot Analysis

An approximately 50-mg sample of frozen tissue was homogenized in a Dounce homogenizer using 500 μl lysis buffer (20 mM Tris HCl, pH 7.4, 150 mM NaCl, 25 mM NaF, 1% NP40, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol, 1 mM $NaVO_4$, and one protease inhibitor pill [Roche Applied Scientific, Indianapolis, IN, USA]/10 ml), followed by a 5-s treatment with a sonic dismembrator (Fisher Scientific, Pittsburgh, PA, USA). Insoluble material was removed by centrifugation at 13,000 × *g* for 15 min at 4°C. Protein concentration was estimated by the method of Lowry, and equal amounts of total protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were transferred to polyvinyl difluoride membranes, and blocked in 10 ml 5% milk in Tris-buffered saline containing 1% Tween-20 (TBST). Both the PTEN antibody (ABM-2052; Cascade Bioscience, Winchester, MA, USA) and the β-actin antibody (A-5441; Sigma-Aldrich, St. Louis, MO, USA) were used at 1:5,000 dilution in TBST. Proteins were visualized using enhanced chemiluminescence (Amersham, Biosciences, Piscataway, NJ, USA).

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were digested with protease XXV (Lab Vision Corp., Fremont, CA, USA) at 37°C for 10 min, and peroxidase activity was quenched with 3% hydrogen peroxide in phosphate-buffered saline. Sections were incubated with an antibody recognizing the Ser-473 phosphorylated form of PKB/Akt (from Cell Signaling Technology, Inc., Danvers, MA, USA; used at 1:50 dilution) and PTEN (from Cell Signaling Technology, Inc.; used at 1:1,000

Table 1. *PTEN* exon primers used for PCR

Exon	Sequence
Exon 1F	5'-GCAGCTTCTGCCACTTCTCT-3'
Exon 1R	5'-CATCCGTCTACTCCCACGTT-3'
Exon 2F	5'-CTCCAGCTATAGTGGGGAAAA-3'
Exon 2R	5'-CTGTATCCCCCTGAAGTCCA-3'
Exon 3F	5'-TGGTGGCTTTTTGTTTGTTT-3'
Exon 3R	5'-CATGAATCTGTGCCAACAATG-3'
Exon 4F	5'-AAAGATTCAGGCAATGTTTGTT-3'
Exon 4R	5'-TCTCACTCGATAATCTGGATGAC-3'
Exon 5F	5'-GGAATCCAGTGTTTCTTTTAAATACC-3'
Exon 5R	5'-TCCAGGAAGAGGAAAGGAAAA-3'
Exon 6F	5'-ATGGCTACGACCCAGTTACC-3'
Exon 6R	5'-TTGGCTTCTTTAGCCCAATG-3'
Exon 7F	5'-TGCTTGAGATCAAGATTGCAG-3'
Exon 7R	5'-GCCATAAGGCCTTTTCCTTC-3'
Exon 8F	5'-TGTCATTTCATTTCTTTTTCTTTTC-3'
Exon 8R	5'-AAGTCAACAACCCCCACAAA-3'
Exon 9F	5'-TGTTCATCTGCAAAATGGAAT-3'
Exon 9R	5'-CAAGTGTCAAAACCCTGTGG-3'

Abbreviations: F, forward; B, reverse.

dilution) at 4°C overnight. Primary antibody incubations were followed by incubation with biotinylated secondary antibody (1:200; no. BA-2000; Vector Labs, Burlingame, CA, USA) and avidin-biotin complex (1:100; no. PK6100; Vector Labs Vectastain ABC Kit) for 30 min each. Staining was visualized using 3,3'-diaminobenzidine tetrahydrochloride, and slides were counterstained with hematoxylin. Sections from nontumor brain and a *PTEN*-mutant GBM were used as controls for both phospho-PKB/Akt and PTEN. Staining for PKB/Akt was recorded as negative or positive in tumor cells. Staining for PTEN was recorded as positive and negative in tumor cells and in comparison to both normal neuronal and vascular cells and the positive control slide.

Results

Whereas DNA isolated from nontumor brain samples revealed no evidence of methylation of the *PTEN* promoter, DNA from 10 of 15 (67%) grade II OAs, 6 of 14 (43%) grade II astrocytomas, 4 of 8 (50%) grade II oligodendrogliomas, and 2 of 23 (9%) de novo GBMs displayed robust methylation at this region (Fig. 1A, Table 2). These differences in *PTEN* methylation frequencies among all five groups, as well as all four tumor groups, were highly statistically significant ($p < 0.001$, Fisher's exact test). In addition, pairwise comparisons between de novo GBMs and the other three tumor histologies were statistically significant. A comparison of the three low-grade tumors did not show statistically significant differences (Table 2).

We next examined whether methylation at the *PTEN* promoter affected the expression levels of PTEN protein in grade II astrocytomas and OAs. We analyzed PTEN expression by both Western blot analysis and immunohistochemistry (IHC), using sections of nontumor brain and GBM as a positive and negative control, respectively. Although nontumor brain showed strong staining, and the GBM showed negative staining by both techniques, neither approach showed a correlation between *PTEN* methylation and PTEN levels. Western blotting indicated that PTEN expression was uniformly high in 20 grade II tumors examined, regardless of *PTEN* methylation (data not shown). We hypothesized that this could be due to the infiltrative nature of low-grade glioma resulting in the contamination of normal tissue in the tumor sections analyzed. We therefore also analyzed PTEN levels by IHC to determine if regions of lower PTEN staining correlated with the presence of tumor material. This analysis showed that the majority of low-grade tumors

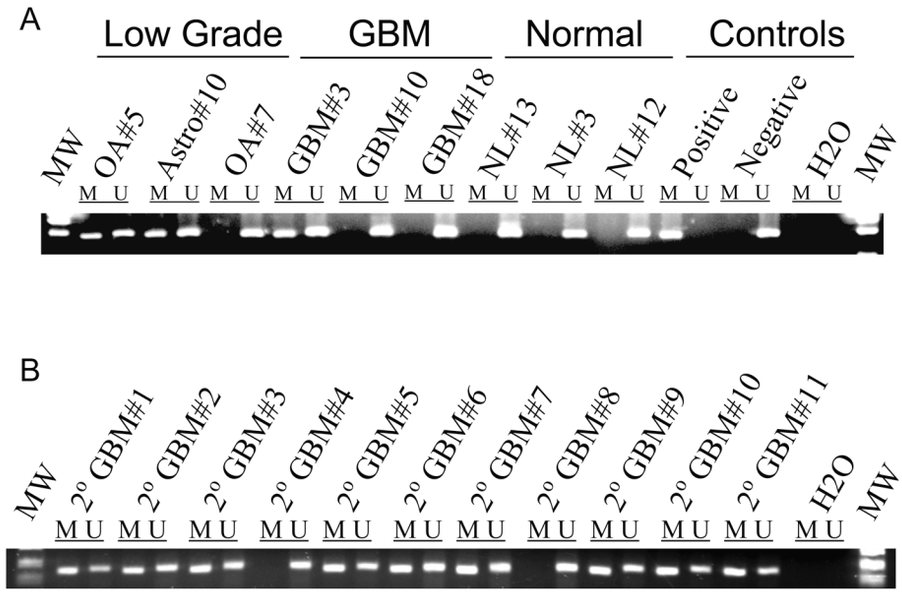

Fig. 1. (A) Methylation analysis of the *PTEN* promoter region by methylation-specific PCR. DNA from the indicated tumor samples and from normal tissues was treated with sodium bisulfite and subjected to PCR with methylation- and unmethylation-specific primers. All methylation-positive brain tumor tissues, oligoastrocytoma (OA) 5, astrocytoma (Astro) 10, and glioblastoma multiforme (GBM) 3, demonstrated both methylated and unmethylated products, indicating the likelihood of normal cell contamination. The negative brain tumor tissues and normal brain tissues demonstrated unmethylated products only. MW, molecular weight marker; M, methylated; U, unmethylated. (B) Methylation analysis of the *PTEN* promoter in secondary GBM. DNA from 11 secondary GBM tumors was isolated and treated with sodium bisulfite. PCR was performed using methylation- and unmethylation-specific primers, and the products were separated by electrophoresis. Abbreviations: MW, molecular weight marker; M, methylated; U, unmethylated.

Table 2. *PTEN* methylation in nontumor brain tissue and gliomas

Histology	Number Methylated (%)	Number Not Methylated (%)
Nontumor brain ($n = 13$)	0 (0)	13 (100)
Grade II oligoastrocytomas ($n = 15$)	10 (67)	5 (33)
Grade II astrocytomas ($n = 14$)	6 (43)	8 (57)
Grade II oligodendrogliomas ($n = 8$)	4 (50)	4 (50)
Grade III astrocytomas ($n = 19$)	13 (68)	6 (32)
De novo GBM ($n = 23$)	2 (9)	21 (91)
Secondary GBM ($n = 11$)	9 (82)	2 (18)

Comparisons of methylation status yielded the following p-values (based on Fisher's exact test or its extension [Fisher-Freeman-Halton]): all seven tissue categories, $p < 0.001$; the six tumor types, $p < 0.001$; de novo GBM samples versus grade II tumors, $p < 0.001$; the four tumor types that were initially low-grade tumors (low-grade plus secondary GBM), $p = 0.22$; de novo GBMs versus secondary GBMs, $p < 0.001$.

Table 3. Association of *PTEN* methylation and phospho-PKB/Akt status in low-grade astrocytomas and oligoastrocytomas

Tumor	*PTEN* Methylation	PKB/Akt Phosphorylation	PTEN IHC Staining
OA 9	–	–	None
OA 10	+	+	None
OA 11	+	+	Focal
OA 12	+	–	None
OA 13	–	–	None
OA 14	–	–	None
Astro 1	–	+	None
Astro 2	–	–	Diffuse
Astro 3	+	+	Diffuse
Astro 4	+	+	None
Astro 5	–	–	None
Astro 6	+	–	Focal
Astro 7	+	+	None
Astro 8	–	–	None
Astro 9	–	+	Focal
Astro 10	+	+	Diffuse
Astro 11	+	+	Diffuse
Astro 12	–	–	None

Abbreviations: Astro, astrocytoma; IHC, immunohistochemistry; PKB/Akt, protein kinase B; OA, oligoastrocytoma.

analyzed (17 of 21) actually showed reduced PTEN staining in the tumor cells, with only four tumors showing diffusely positive staining, but this did not correlate with *PTEN* methylation (see Table 3 for the tumors in which PKB/Akt phosphorylation was also analyzed).

We therefore turned to a sensitive assay for alterations in PTEN activity and assessed activity of PKB/Akt, a downstream kinase regulated by PTEN. As a measure of PKB/Akt activity, we performed IHC analysis using an antibody specific for the Ser-473 phosphorylated form of PKB/Akt. Paraffin-embedded tissues were available for six of the grade II OAs and 12 of the grade II astrocytomas, and these tumor sections were scored blindly by a neuropathologist for the presence or absence of PKB/Akt phosphorylation. Fig. 2 shows two representative low-grade tumors with extensive Ser-473–PKB/Akt phosphorylation and two tumors negative for phospho-PKB/Akt staining. Table 3 reveals the correlation between methylation status and phospho-PKB/Akt staining in the 18 grade II astrocytomas and OAs. Our analysis shows that of these 18 low-grade tumors, nine were positive for *PTEN* methylation (50%) and nine were positive for PKB/Akt phosphorylation (50%). Therefore, if there were no correlation between *PTEN* methylation and PKB/Akt phosphorylation, the expected concordance would be 0.5 (25% both positive + 25% both negative). In fact, 14 of 18 samples showed concordance between *PTEN* methylation and PKB/Akt phosphorylation, generating a point concordance of 0.78 (95% confidence interval, 0.53–0.92), indicating a statistically significant correlation between *PTEN* methylation and PKB/Akt phosphorylation ($p = 0.028$, one-tailed Fisher's exact test).

Given the high frequency of *PTEN* methylation in grade II gliomas, we hypothesized that upon progression to higher grade gliomas, grade III tumors and secondary GBMs would retain this epigenetic aberration and display frequent *PTEN* methylation, in contrast to de novo GBMs in which *PTEN* methylation would prove rare. We obtained 19 grade III gliomas (10 OAs and 9 anaplastic astrocytomas) and 11 secondary GBM samples from patients who had pathologically documented prior low-grade gliomas, and found strong methylation of the *PTEN* promoter in 12 of 19 grade III gliomas and 9 of 11 secondary GBMs (Table 2, Fig. 1B). These frequencies are significantly different compared to the frequency

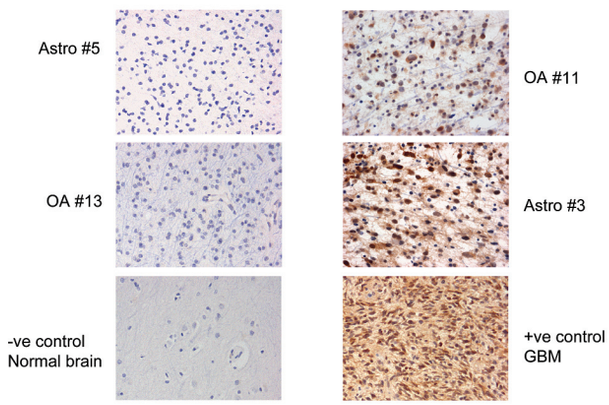

Fig. 2. Immunohistochemical staining for phospho-PKB/Akt in low-grade astrocytomas and oligoastrocytomas. Eight-micrometer sections from the indicated tumors were stained using a primary antibody recognizing the Ser-473 phosphorylated form of PKB/Akt. All images are at the same magnification (magnification ×200). The left column demonstrates negative staining in a grade II astrocytoma (Astro 5) and oligoastrocytoma (OA 13), as well as a section of normal cortex used as a negative control. The right column represents examples of tumors with positive PKB/Akt staining (Astro 3 and OA 11), as well as a glioblastoma multiforme sample with a *PTEN* mutation used as an external positive control.

of *PTEN* methylation in de novo GBMs ($p < 0.001$), but not (with the small number of samples) compared to that seen in low-grade gliomas (Table 2).

Promoter methylation may therefore represent an alternative mechanism of PTEN inactivation. *PTEN* mutation and methylation are likely to be mutually exclusive in any particular tumor. To test this hypothesis, we analyzed 14 of the de novo GBMs and nine of the secondary GBMs (the tumors that had sufficient DNA) for *PTEN* mutations. The analyses of *PTEN* mutations in these tumors showed that none of the nine secondary GBMs harbored *PTEN* mutations. Eight of nine secondary GBMs displayed *PTEN* methylation. In contrast, of the 14 de novo GBMs, four harbored *PTEN* mutations, and these were mutually exclusive with the two tumors that displayed *PTEN* methylation. Three *PTEN* mutations were in exon 5, and one was in exon 6, which together compose the core phosphatase domain. Mutations in GBMs consisted of M134I, T131P, R173C, and W111stop. Although the small number of tumors expressing mutant *PTEN* in this analysis precluded a statistically significant conclusion, the data are consistent with the hypothesis that *PTEN* methylation and mutation represent two independent mechanisms of *PTEN* inactivation.

We also analyzed potential associations of *PTEN* methylation and PKB/Akt phosphorylation with progression-free survival in the low-grade glioma patients. No statistically significant associations with clinical outcome were found. For the methylation analysis, we had information on 18 patients, of whom 10 had progressed. The hazard ratio for the difference was 0.28 ($p = 0.06$; 95% confidence interval, 0.07–1.13) favoring patients whose tumors were methylated. For PKB/Akt phosphorylation, only 12 patients had the necessary information, and 7 of these 12 had progressed. The hazard ratio for this difference was 0.32 ($p = 0.16$; 95% confidence interval, 0.06–1.69) favoring those with phosphorylation.

Discussion

Methylation of the *PTEN* promoter occurs in a number of human tumor types, including endometrial,[24] gastric,[25] brain,[26] breast,[27,28] ovary,[29] cervical,[30] lung[22,31] colorectal,[32] and pancreatic[33] cancers; thyroid[34,35] and soft tissue[36] sarcomas; and melanoma.[37] Interestingly, these studies have documented *PTEN* methylation within three separate regions of the *PTEN* promoter, depicted in Fig. 3. Studies of endometrial, gastric, pancreatic, and cervical cancers used methylation-specific primers that recognize a region (Fig. 3, region a) approximately 200 nucleotides upstream of the translational start site that shares 100% identity with the *PTEN* pseudogene on chromosome 9. Zysman et al.[38] propose that methylation seen with these primers represents amplification of the *PTEN* pseudogene, and a survey of endometrial cancers in their study supports this notion. However, the bona fide *PTEN* gene can also show methylation in this region, as demonstrated by a study in GBMs using primers specific for *PTEN* rather than the *PTEN* pseudogene. Methylation of this region of the *PTEN* promoter was demonstrated in 21 of 31 GBMs.[26] A separate region within the *PTEN* promoter (Fig. 3, region b), approximately 800 nucleotides upstream of the translational start site, exhibited methylation in 43 of 90 breast cancer specimens.[27] Regions a and b lie within the 5'-untranslated sequence of *PTEN* mRNA that extends more than 1,000 bases upstream of the translational start site. Yet a third region of methylation (Fig. 3, region c), located approximately 2.4 kilobases upstream of the *PTEN* translational start site, was documented in 7 of 20 non–small-cell lung carcinomas,[22] as well as 6 of 47 soft tissue sarcomas.[36] Our studies of gliomas focused on regions b and c. We found no methylation in region b in the first 30 GBM and anaplastic astrocytoma samples analyzed, so we turned our attention to the upstream region c located 2.4 kilobases from the initiating AUG. In this study, the first to examine this region within CNS tumors, we found region c to be frequently methylated in low-grade tumors and secondary GBMs. It should be noted that only one study[37] has demonstrated both reactivation of *PTEN* by the demethylating agent 5'-azacytidine and concomitant reversal of *PTEN* promoter hypermethylation. That study examined region c, which we have focused on in our investigation. Several but not all studies have shown reactivation of the *PTEN* gene by 5'-azacytidine but have not measured CpG methylation following treatments. Hence, while the promoter regions most relevant to methylation-related gene silencing of *PTEN* are not fully known, the region we examined appears to have biological relevance to *PTEN* regulation. We also found low frequency of methylation of the *PTEN* pseudogene at region a in samples of nontumor brain, low-grade gliomas, and de novo GBMs (data not shown). This did not correlate with either PTEN expression or PKB/Akt phosphorylation, and given the lack of indication of functional significance of methylation of this region, we did not pursue it further. Thus, tumor-associated methylation of the *PTEN* promoter is highly complex, occurs in three distinct regions upstream of the translational start site, and is likely dependent on both cell type and tumor grade.

The best understood mechanism for inactivating *PTEN* is through mutation, which occurs relatively frequently in de novo GBMs, occasionally in secondary GBMs, and exceedingly rarely in low-grade gliomas. Conversely, our present analysis found that *PTEN* methylation is common in grade II gliomas, scarce in de novo GBMs, and very frequent in secondary GBMs. Whereas *PTEN* mutations have proven important in the pathogenesis of de novo GBMs, *PTEN* methylation may play a key role in the development of low-grade gliomas and their subsequent progression to secondary GBMs. Thus, the two GBM subtypes share in their genesis activation of the PI3K pathway, but through distinct mechanisms. Discordance between *PTEN* methylation and *PTEN* mutations found in the GBM samples we examined supports the hypothesis that both of these genetic alterations underlie glioma development and can generate similar malignant phenotypes. In an earlier study,[26] methylation of *PTEN* occurred in several tumors that showed *PTEN* mutations and/or *PTEN*

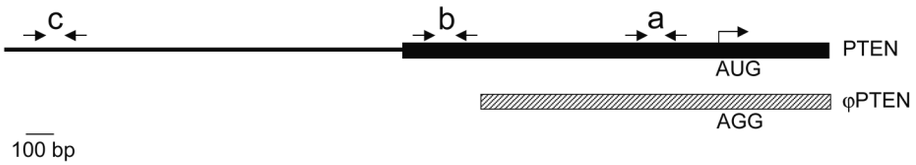

Fig. 3. Schematic diagram depicting regions of methylation demonstrated for *PTEN* and *PTEN* pseudogene (φPTEN). The black elongated rectangle represents the *PTEN* transcribed region, and the initiating methionine codon is indicated. The black line extending to the left of the rectangle represents the *PTEN* promoter. The hatched rectangle represents the *PTEN* pseudogene sequence on chromosome 9 showing the region of high homology to the *PTEN* transcript. Tumor-associated *PTEN* methylation has been reported in regions a,[24–26,30] b,[27] and c.[22] Methylation of the *PTEN* pseudogene has been demonstrated in Zysman et al.[38]

LOH. In contrast, our study did not show tumors with concurrent *PTEN* promoter methylation and gene mutations, although the small numbers precluded statistically significant conclusions. One possible reason for this discrepancy could be the different regions of the PTEN promoter assessed (region a in Baeza et al.[26] and region c in the present study). Alternatively, the GBMs analyzed in Baeza et al.[26] might not have been clonal and may represent distinct tumor populations. Finally, because the *PTEN* LOH has previously been shown to be frequent in secondary GBMs,[10,39] we hypothesize that methylation and LOH could underlie loss of PTEN function in secondary brain tumors, whereas mutation and methylation or mutation and LOH could account for loss of PTEN function in primary GBMs. Expanding our analysis to greater numbers of tumors should clarify this. We have also not yet established from our studies whether methylation accounts for the loss of function of one or both *PTEN* alleles. Interestingly, LOH of 10q is infrequent in low-grade gliomas[10,39] suggesting that *PTEN* methylation may only occur on one allele. *PTEN* may thus be acting in a haploinsufficient manner in low-grade gliomas to give moderate increases in PI3K signaling, and loss of the second allele in GBMs increases activity further. Allele-specific MSP would be required to test this hypothesis.

PTEN methylation in grade II gliomas correlated with PKB/Akt activation but did not correlate with PTEN expression levels as measured by either Western blot or IHC. Although some studies have seen a correlation between *PTEN* promoter methylation and PTEN expression,[32,35] the correlations are not perfect and, in some cases, require tissue microdissection.[26] Other studies have also noted no correlation between *PTEN* methylation and PTEN expression.[31] Western blot analysis suggested that PTEN levels were uniformly high in the tumor samples, perhaps reflecting the invasive nature of low-grade gliomas, resulting in extensive normal tissue contamination within tumor specimens. Whereas contaminating normal tissue likely masks small reductions in PTEN expression by Western blot, the sensitive methylation assay used herein can detect methylation in a single *PTEN* allele, in samples containing only small proportions of tumor cells. In addition, as mentioned above, *PTEN* methylation may decrease expression in only a single allele in low-grade gliomas, thereby confounding detection of small decreases in expression. In contrast, very low levels of PKB/Akt phosphorylation in normal brain tissue[16] facilitate detection of even small increases in PKB/Akt phosphorylation in tumor specimens, which allows visualization of this functional consequence of *PTEN* methylation. Conversely, IHC analysis suggested that most of the tumor cells present had low levels of PTEN staining, with only 4 of 21 tumors analyzed scored as having diffusely positive immunoreactivity. Regardless of whether this reflects that the PTEN IHC staining is not reliable in our hands (although it was performed twice independently in two different laboratories) or that PTEN levels in the low-grade tumors are decreased for multiple reasons, of which *PTEN* methylation is only one, it is clear that PTEN levels did not correlate with PKB/Akt phosphorylation (Table 3). Therefore, whether there is a simple linear relationship among *PTEN* methylation, decreased PTEN expression, and increased PKB/Akt phosphorylation remains to be proven. Nevertheless, *PTEN* methylation appears to be a marker for low-grade tumors that show increased PKB/Akt phosphorylation.

These technical difficulties for measuring PTEN levels aside, the role of promoter methylation in decreasing gene expression during tumorigenesis remains controversial.[40] There is no clear mechanism leading to de novo methylation of CpG islands in somatic glial cells and resultant selective decreased expression of certain tumor suppressor proteins such as PTEN. Alternatively, promoter methylation may reflect gene inactivity rather than represent a primary cause of transcriptional repression. Although such mechanistic questions remain to be answered, *PTEN* methylation in human malignancies raises the possibility of using agents designed to antagonize CpG hypermethylation, for example, 5-azacytidine and 5-aza-2'-deoxycytidine (also known as Vidaza and decitabine, respectively). Vidaza has been approved for myelodysplastic syndrome[41] and is currently in clinical trials for non–small-cell lung cancer and prostate cancer.[42] Potential side effects induced by global demethylation notwithstanding, if methylation is a consequence of silencing rather than a cause, then demethylation may not restore gene activity. Of note, however, are studies in which treatment of a prostate cancer xenograft,[43] a lung

cancer cell line,[22] and two melanoma cancer cell lines[37] with decitabine resulted in increased PTEN expression. These latter two studies documented *PTEN* methylation at the same region of the promoter as analyzed in our studies, adding credence to the potential treatment of low-grade gliomas with such demethylating agents.

Although the small sample size precluded statistically significant conclusions, it was unexpected that *PTEN* methylation and increased PKB/Akt phosphorylation were associated with better outcome in low-grade gliomas. While decreased levels of PTEN generally portend poor clinical outcome in GBM[16] and other tumors,[44] there are reports of *PTEN* mutations correlating with favorable outcome in endometrial and lung cancer.[31,45,46] This observation has been interpreted as *PTEN* alterations being required for tumor-associated initiating lesions, with alternative pathways being more important for tumor progression. Such correlations between genetic alterations and favorable prognosis in gliomas have also been noted for *TP53* mutations[47] and loss of chromosome 1p/19q.[48] Clearly, further work is required to dissect the significance of differing *PTEN* genetic alterations in the establishment and development of human gliomas.

In addition to demethylating agents, our results point to the potential utility of inhibitors of the mammalian target of rapamycin (mTOR) in the treatment of low-grade gliomas. Rapamycin and its esterified analogues RAD-001 and CCI-779 have favorable toxicity profiles and carry additional appeal since tumors that express activated PKB, as is evident in the low-grade gliomas and secondary GBMs described herein, appear particularly sensitive to mTOR inhibition.[49,50] Finally, studies that describe detection of tumor-associated gene methylation in peripheral blood samples from cancer patients[51] further increase enthusiasm for future patient selection for treatment with these and other therapies.

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